13

Studies on the Histology and Histopathology of the Rainbow Trout, *Salmo gairdneri irideus*. I. Hematology: Under Normal and Experimental Conditions of Inflammation[1]

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(Plate I)

MOST studies in comparative hematology have been limited to descriptions of the staining properties of fixed cells. The present study was made on living blood cells of the rainbow trout, *Salmo gairdneri irideus,* using phase contrast microscopy, to further elucidate physiological as well as morphological changes under experimental conditions. Such changes were compared with similar effects reported in more common laboratory animals.

The circulatory system of the rainbow trout is sensitive to foreign stimuli and reflects the homeostasis of the animal. Changes in the blood picture were used, therefore, as criteria of systemic response to experimental conditions. Before such changes could be evaluated, the normal blood picture had to be determined and a standard established.

Descriptive studies of teleost blood cells of various species have included perch (Yokoyama, 1947), carp and brook trout (Dombrowski, 1953) and salmon (Watson *et al.*, 1956). Detailed reports on rainbow trout have not been found. The response of leukocytes to various irritants has been described for many laboratory animals including dogfish shark (Reznikoff & Reznikoff, 1934), turtle (Charipper & Davis, 1932; Ryerson, 1943), perch (Yokoyama, 1947), mice and rats (Harlow & Selye, 1937). The influence of adrenal cortical hormones and ACTH on circulating leukocytes in mice, rats and rabbits has also been described (Dougherty & White, 1944; Palmer *et al.*, 1951). The relation among leukocytes, ACTH and adrenal cortex was reviewed by Sayers (1950).

Although the blood-forming centers in fish differ from those found in mammals, and the individual cells vary betwen the classes, the author is retaining such terms as myeloid, myelocyte and lymphoid in order to be consistent with literature on fish hematology and to describe the various stages in granulocyte maturation in the fish as compared to similar well-defined stages in mammals. The prefix myelo- in this text is used not in reference to bone marrow, but to granulocytic as distinct from lymphoid elements.

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Normal Blood Picture

Materials and Methods

The animals used for all studies were from the same hatching group (October 9, 1953). Fish were delivered from the Nevins State Fish Hatchery, Madison, Wisconsin, to the University of Wisconsin Lake Laboratory and maintained in oxygenated water at 12-12.5°C. for several days so they might become acclimated to the experimental tanks. The trout, of both sexes, averaged 15 cm. fork length and 50 gm. weight. The age ranged from 12 months at the start of this study to 21 months at its completion.

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Since the variability of cell counts is great, each experimental group consisted of animals kept under the same conditions and of the same age and approximate size. After severing the tail, blood samples were collected from the haemal vessels, using no anticoagulants, and each animal was autopsied, with particular attention being paid to the hemopoietic structures. All trout used were in normal condition as far as could be determined.

Living blood cells were studied and counted by phase contrast microscopy, using a dark contrast-medium oil immersion objective. Wright's stained smears were used for comparison (Plate I). The former method was found to afford greater accuracy of identification and precision in counting, the artefacts of staining and loss of fragile heterophils being minimal. Total red and white cell counts were made (Yokoyama, 1947), in order to determine the total number variability in different animals under normal and experimental conditions. Since the erythrocyte count did not exhibit significant change, it was used as the basis for the differential leukocyte counts: all leukocytes within the range of 300 red blood cells were counted. The cells counted included heterophils, lymphocytes and thrombocytes; the number of eosinophils and basophils was negligible, and monocytes were absent.

Results

The criteria for cell identification in phase studies include size, shape, degree of motility, nuclear-cytoplasmic ratio, nuclear size and shape, chromatin pattern, cytoplasmic granulation and mitochondria.

The mature erythrocyte is oval and nucleated, with abundant cytoplasm containing many very small, motile mitochondria. The smaller, rounder polychromatophilic erythrocyte differs in having less hemoglobin and a heavier nuclear membrane, similar to that of the lymphocyte. Lymphocytes are the most abundant, being smaller and rounder than the red cell, often exhibiting pseudopod formation. The round or slightly indented nucleus is surrounded by a thin rim of cytoplasm with motile, rod-shaped mitochondria. The outstanding feature of the lymphocyte is the nuclear pattern; the chromatin forms a distinct network of alternately dark and highly refractile, light areas.

In the highly amoeboid heterophil, the partially lobed nucleus is in almost continuous rolling motion, while, after staining, the nucleus is lobed or ribbon-like. The cytoplasm, which exhibits continuous streaming and pseudopod formation, is filled with granules and filamentous mitochrondia. Cells comparable to myelocytes and metamyelocytes are not uncommon and may be distinguished from the mature heterophil by being less motile and round to oval in shape, with a more rounded nucleus and less granulation. The myelocyte is smaller and more rounded than either of the older cells. In both immature cell types the nucleus exhibits a folding motion with wrinkling of the surface, rather than the distinct roll of the heterophil.

Although thrombocytes are usually in the blebbed state, intact cells are visible. Young cells are similar to lymphocytes, although smaller and rounder. Mature thrombocytes are elongated. In contrast to the lymphocyte, the cell may be identified by its indistinct chromatin network, paucity of cytoplasmic granules and barely motile mitochondria.

Occasional eosinophils and basophils are seen. Eosinophils are slightly larger than lymphocytes, with numerous, extremely refractile, spherical granules. The basophil, which is the largest leukocyte, has an eccentric spherical nucleus, indistinct chromatin and a very prominent nucleolus. The large, round cytoplasmic granules are characterized by an internal, laminated or striated pattern.

Macrophages, which are not usually found in a normal blood smear, are prominent in bacterial and other infections. These cells are the largest in the circulation, being twice the size of the basophil. They contain abundant debris.

Leukocyte counts were made on 10 normal rainbow trout. The number of fish leukocytes, particularly lymphocytes and thrombocytes, varies. In order to establish a normal standard, the mean ± standard error of mean (S.E.) and the range of mean, where range is the distance between the upper and lower 95% confidence limits, were determined for each cell type. The mean ± S.E. and range of mean for the heterophil, lymphocyte and thrombocyte are 2.3 ± 0.66 (0.98-3.62), 20.5 ± 2.31 (15.88-25.12) and 11.1 ± 2.33 (6.44-15.76), respectively.

Effects of Turpentine, Cortisone and ACTH on Leukocytes

Materials and Methods

Trout were injected intraperitoneally under the pelvic fin with 0.2 cc./100 gm. of turpentine, N.F. Controls were given injections of 0.6 cc./100 gm. of Ringer-Locke solution (0.65%). The animals were divided into two series; the first was sacrificed after 1, 3, 5, 7 and 24-hour intervals, the second after 1, 3, 5, 6, 7, 10, 24, 48, 60 and 72-hour intervals. Differential leukocyte counts were made after each time interval. Total

red and white cell counts were also made 6 and 24 hours after turpentine injection.

To study the effect of cortisone on leukocyte response, trout were divided into two series; each received 0.2 cc./100 gm. of turpentine. Those in series 1 were given intraperitoneal injection of cortisone (Cortone Acetate, Merck and Co.) in saline suspension concurrently with the turpentine, while series 2 received cortisone 24 hours in advance of the turpentine. Animals were subdivided into dosage groups, receiving 0.3, 0.6 and 1.0 mg./cc., respectively. Controls were injected with 1.0 mg./cc. of cortisone without turpentine. Blood samples were taken 6 and 24 hours after turpentine injection as well as after the control cortisone injection.

To determine whether administration of ACTH would stimulate adrenal tissue to secrete a hormone eliciting the same leukocyte response as cortisone, another group of trout was injected with ACTH (Corticotropin, ACTH, Armour Laboratories) in Ringer-Locke solution. Dosages of 2 U. S. P. units (I. U.) were used and blood samples taken after 6 and 24 hours.

Leukocyte counts were made and the 95% range of the mean in normal and experimental trout compared. Data were considered statistically significant when two ranges failed to overlap.

Results

Injections of Ringer-Locke solution resulted in no significant change in either heterophil or lymphocyte counts. The slight increase in heterophils encountered in a few animals was attributed to handling and other unknown factors. Turpentine injection elicited a marked response. Total red cell counts did not differ significantly from normal, while total leukocyte counts were notably increased. Leukocyte counts of the first series, over a 24-hour period, are given in Table 1; those for series 2, over a 72-hour period, in Table 2.

In response to injected turpentine, the heterophil count rose significantly within 5 hours, reached its peak at 6 hours and remained high for 60 hours. The lymphocyte count dropped significantly in 6 hours, and remained low for 72 hours. No significant change occurred in thrombocyte number in the first 24 hours, but decreases occurred at 48 and 60 hours. The early increase in heterophils is mainly due to release of myelocytes and metamyelocytes into the circulation.

The mortality rate following turpentine injection averaged 30%, the greatest loss occurring between the second and fourth post-injection hours.

Leukocyte counts of trout following cortisone and concurrent injections of turpentine and cortisone are listed in Table 3. Counts taken after prior injections of cortisone are given in Table 4.

Cortisone alone had little effect on the heterophil count; the change in lymphocyte count, however, was marked. Lymphopenia was noted 6 and 24 hours after injection, accompanied by thrombocytopenia. Cortisone, when given concurrently with turpentine, resulted in less heterophilia than did turpentine alone, the increase being due to a preponderance of mature heterophils. The effect of cortisone was most apparent when injected in advance of the irritant. Although the largest dose yielded the maximum effect, little difference existed between dosage groups.

After prior injection of cortisone, lymphopenia and thrombocytopenia were greater than that noted after concurrent injections. A comparison of mortality rates indicates that cortisone has a significant effect. Concurrent injections in series 1 maintained the average (30%) mortality rate. However, prior injections in series 2 average only 16%, a reduction of almost one-half.

ACTH injection resulted in lymphopenia and thrombocytopenia, as did cortisone. In addition, heterophilia, due to increased mature cells, resulted. Leukocyte counts are given in Table 5.

Discussion

The blood response elicited in trout by turpentine is similar to that reported in other animals, including the dogfish (Reznikoff & Reznikoff, 1934), turtle (Ryerson, 1943), chicken (Bradley, 1937) and perch (Yokoyama, 1947). Comparable effects were also reported in mice and rats after injections of adrenalin or formaldehyde (Harlow & Selye, 1937).

The response of the trout was not limited to the irritant, but was also an expression of the shock reaction to the toxin. The high mortality seen in the first hours is attributed to this. The leukocyte response to pituitary and adrenal cortical hormones in trout is comparable to that noted in mammals. A decrease in leukocytosis (due to neutrophilia) was reported by Palmer *et al.* (1951) in turpentine-injected rats after administration of cortisone and ACTH, with the greater reduction following cortisone. The time relationship between administration of cortisone and turpentine was more important than cortisone dosage. Maximal inhibition of inflammation was obtained after prior injection of cortisone, which permitted adequate time for absorption. The effect on mortality also appears to

be dependent upon absorption sufficient to inhibit shock.

The heterophilia noted in trout after administration of ACTH is similar to the neutrophilia elicited by ACTH in intact and adrenalectomized rats (Palmer *et al.*, 1951) and in rats, mice and rabbits following ACTH or foreign protein (Dougherty & White, 1944). The neutrophil or heterophil, therefore, is not under direct adrenal cortical control, but is subject to various influences.

Lymphopenia following cortisone or ACTH is a more specific response. This reaction, reported absent after adrenalectomy or injection of other proteins (Dougherty & White, 1944), results from many unrelated stimuli, including turpentine, and is due to increased adrenal cortical activity initiated by ACTH. Decreased lymphocyte number with stress or hormone treatment in intact animals appears to follow adrenal cortical inhibition of the lymphoid organs.

In rainbow trout, lymphopenia and thrombocytopenia resulted from stress, cortisone and ACTH, while heterophilia followed stress and ACTH injection. Therefore, the mechanism of leukocyte control, as well as the physiological response of each cell type, in the trout is comparable to that in the mammal. Since significant heterophilia was not caused by cortisone, it is inferred that granulocyte-forming centers are not under adrenal cortical control, as may be the case with lymphocytes and thrombocytes. In the trout, where granulocyte- and agranulocyte-forming tissue are located in the same hemopoietic organs, multiple controls exist.

Summary

1. Living blood cells from rainbow trout are described, and differential counts made, using phase contrast microscopy. In normal blood the predominant leukocyte is the lymphocyte, the heterophil is scarce, and eosinophils and basophils are seen only occasionally.
2. Changes in blood picture are used as criteria of systemic response to experimental conditions; the effects of turpentine, cortisone and ACTH on the leukocyte counts are determined.
3. Turpentine produced a sterile inflammation, resulting in heterophilia, lymphopenia and thrombocytopenia. Blood counts, made over a 72-hour period, revealed marked heterophilia at 6 hours due to release of myelocytes and metamyelocytes.
4. Cortisone, given concurrently and in advance of turpentine, reduced the inflammatory

Table 1. Leukocyte Counts over 24-hour Period Following Injection of 0.2 cc./100 gm. Turpentine

Time interval (hr.)	Heterophil			Lymphocyte			Thrombocyte		
	n	Mean ± S.E.	95% Range	n	Mean ± S.E.	95% Range	n	Mean ± S.E.	95% Range
1	10.0	8.5 ± 1.5	5.5-11.5	21.0	20 ± 1	18-22	12.0	13 ± 1	11-15
	7.0			19.0			14.0		
3	14.0	16.5 ± 2.5	11.5-21.5	13.0	19 ± 6	7-31	5.0	15.5 ± 10.5	0-36.5
	19.0			25.0			26.0		
5	30.0	22.5 ± 7.37	7.76-37.24	25.0	21.5 ± 3.5	14.5-28.5	31.0	17.5 ± 13.5	0-44.5
	15.0			18.0			4.0		
7	14.0	12.0 ± 2	8-16	4.0	6 ± 2	2-10	4.0	5 ± 1	3-7
	10.0			8.0			6.0		
24	19.0	14.0 ± 5	4-24	7.0	3.5 ± 3.5	0-10.5	12.0	11.5 ± 0.05	11.4-11.6
	9.0			0			11.0		

TABLE 2. LEUKOCYTE COUNTS OVER 72-HOUR PERIOD FOLLOWING INJECTION OF 0.2 CC./100 GM. TURPENTINE

Time interval (hr.)	Heterophil			Lymphocyte			Thrombocyte		
	n	Mean ± S.E.	95% Range	n	Mean ± S.E.	95% Range	n	Mean ± S.E.	95% Range
1	3.0 7.0 0 4.0	3.5 ± 1.44	0.62-6.38	21.0 11.0 2.0 8.0	10.5 ± 3.98	2.54-18.46	8.0 8.0 15.0 11.0	10.5 ± 3.24	4.02-16.98
3	17.0 1.94 5.0 22.0	11.49 ± 4.98	1.53-21.45	17.0 17.48 2.0 20.0	14.24 ± 3.98	6.28-22.2	6.0 10.68 6.0 6.0	7.17 ± 1.32	4.53-9.81
5	15.0 14.0 22.04 22.0	18.26 ± 2.18	13.9-22.62	18.0 2.0 9.27 8.0	9.32 ± 3.29	1.74-16.9	3.0 17.0 3.83 1.0	6.21 ± 3.65	0-13.51
6	24.0 43.0 33.0 15.24	28.81 ± 4.86	19.09-38.53	7.0 7.0 4.0 0.96	4.74 ± 1.45	1.84-7.64	7.0 10.0 0 6.67	5.92 ± 2.11	1.7-10.12
7	34.2 15.0 20.0 16.0	21.3 ± 4.43	12.44-30.16	6.84 0 0 1.0	1.96 ± 1.64	0-5.24	10.75 7.0 7.0 11.0	8.94 ± 1.12	6.7-11.18
10	17.36 33.0 15.0 20.0	21.34 ± 2.54	16.26-26.42	0 5.0 2.0 0	1.75 ± 1.49	0-4.73	1.93 10.0 7.0 4.0	5.73 ± 1.76	2.21-9.25
24	11.0 18.0 9.46 9.0	11.87 ± 2.09	7.69-16.05	2.0 2.0 0.95 0	1.24 ± 0.48	0.28-2.2	4.0 10.0 6.62 6.0	6.66 ± 1.25	4.16-9.16
48	6.0 9.68 12.15 6.82	8.66 ± 1.41	5.84-11.48	0 16.45 1.25 1.0	4.68 ± 1.93	0.82-8.54	3.0 9.68 2.80 6.0	5.37 ± 1.26	2.85-7.89
60	5.0 7.81 5.62 25.0	10.86 ± 4.75	1.36-20.36	1.0 7.0 0 6.0	3.5 ± 1.75	0-7	7.0 3.0 6.75 4.0	5.17 ± 1	3.17-7.17
72	16.0 0 3.0 6.0	6.25 ± 4.91	0-16.07	2.0 0.95 1.0 11.0	3.74 ± 2.43	0-8.57	10.0 10.41 5.0 2.0	6.85 ± 2.03	2.79-10.91

TABLE 3. LEUKOCYTE COUNTS FOLLOWING INJECTION OF 0.2 CC./100 GM. TURPENTINE AND CORTISONE ADMINISTERED CONCURRENTLY

Time interval (hr.) after turpentine	Cortisone dosage mg./cc.	Heterophil			Lymphocyte			Thrombocyte		
		n	Mean ± S.E.	95% Range	n	Mean ± S.E.	95% Range	n	Mean ± S.E.	95% Range
6	(Control, no turpentine)	6.0	3.5 ± 2.5	0- 8.5	1.0	8 ± 7	0-22	6.0	3 ± 3	0-9
	1.0	1.0			15.0			0		
6	0.3	15.57	10.98 ± 4.14	2.7 -19.26	18.51	13.25 ± 5.24	2.77-23.73	9.74	7.27 ± 2.04	3.19-11.35
		6.38			7.98			4.79		
6	0.6	25.0	14.5 ± 5.57	2.86-25.14	7.0	8 ± 1	6-10	14.0	9.33 ± 2.6	4.13-14.53
		10.0			10.0			5.0		
		7.0			7.0			9.0		
6	1.0	11.0	10.18 ± 4.45	1.28-19.08	6.0	6.45 ± 0.67	5.11-7.79	10.0	7.48 ± 2.48	2.52-12.44
		6.71			4.79			1.92		
		25.0			7.0			13.0		
		8.0			8.0			5.0		
24	(Control, no turpentine)	6.0	5 ± 1	3-7	0	2 ± 2	0-6	2.0	3 ± 1	1-5
	1.0	4.0			4.0			4.0		
24	0.3	4.0	–	–	2.0	–	–	2.0	–	–
24	0.6	5.0	–	–	3.0	–	–	1.0	–	–
24	1.0	10.0	7.6 ± 1.51	4.58-10.62	6.0	4.92 ± 0.94	3.04-6.8	0	3.64 ± 2.72	0-9.08
		4.81			5.77			1.92		
		8.0			3.0			9.0		

TABLE 4. LEUKOCYTE COUNTS FOLLOWING INJECTION OF 0.2 CC./100 GM. TURPENTINE AND CORTISONE ADMINISTERED 24 HOURS IN ADVANCE

Time interval (hr.) after turpentine	Cortisone dosage mg./cc.	Heterophil			Lymphocyte			Thrombocyte		
		n	Mean ± S.E.	95% Range	n	Mean ± S.E.	95% Range	n	Mean ± S.E.	95% Range
6	0.3	1.0 8.0 9.0	6 ± 2.18	1.64-10.36	3.0 4.0 4.0	3.67 ± 0.33	3.01-4.33	0 9.0 6.0	5 ± 2.65	0-10.30
6	0.6	10.0 9.0 6.0	8.33 ± 1.2	5.93-10.73	2.0 5.0 4.0	3.67 ± 0.88	1.91-5.43	11.0 10.0 3.0	8 ± 2.52	2.96-13.04
6	1.0	10.0 4.87 10.0 13.0	9.47 ± 1.48	6.51-12.43	6.0 0.97 1.0 3.0	2.74 ± 1.2	0.34-5.14	4.0 1.95 7.0 5.0	4.49 ± 1.27	1.95-7.03
24	0.3	5.0 6.0	5.5 ± 0.5	4.5-6.5	0 0	0	0	1.0 4.0	2.5 ± 1.5	0-5.5
24	0.6	9.0 4.0 2.0	5 ± 2.09	0.82-9.18	3.0 5.0 0	2.67 ± 1.45	0-5.57	3.0 6.0 4.0	4.33 ± 0.88	2.57-6.09
24	1.0	7.0 4.0 10.0	7 ± 1.73	3.54-10.46	2.0 7.0 1.0	3.33 ± 1.86	0-7.05	4.0 6.0 5.0	5 ± 0.57	3.88-6.14

TABLE 5. LEUKOCYTE COUNTS FOLLOWING INJECTION OF 2 U.S.P. UNITS OF ACTH

Time interval (hr.)	Heterophil			Lymphocyte			Thrombocyte		
	n	Mean ± S.E.	95% Range	n	Mean ± S.E.	95% Range	n	Mean ± S.E.	95% Range
6	8.0			10.0			11.0		
	1.88			6.39			7.52		
		7.08 ± 1.88	3.32-10.84		10.53 ± 1.37	6.79-14.27		5.63 ± 1.79	2.05-9.21
	6.42			13.74			0		
	12.0			12.0			4.0		
24	2.0			3.0			5.0		
	3.0			2.0			2.0		
		6.42 ± 2.65	1.12-11.72		2.97 ± 1.09	0.79-5.15		6.24 ± 1.98	2.28-10.20
	6.0			1.0			12.0		
	14.7			5.88			2.94		

response, while advance injection also reduced mortality by almost 50%.

5. Lymphopenia and thrombocytopenia resulted from stress, cortisone and ACTH, while heterophilia followed stress and ACTH injection.
6. Decrease in lymphocyte number appears to be in direct response to adrenal cortical stimulation initiated by ACTH. Heterophilia is not under direct adrenal cortical control, but subject to a wider range of influencing factors.
7. The mechanism of leukocyte control, as well as the physiological response of each cell type, in the trout is comparable to that in mammals.

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EXPLANATION OF THE PLATE

Plate I

Typical fields showing leukocyte distribution in blood of normal and turpentine-injected trout. Note scarcity of heterophils and prevalence of lymphocytes in adjacent fields in normal blood (Figs. 1, 2), compared with reversed condition seen in adjacent fields after turpentine injection (Figs. 3, 4.). Heterophil, **H**; lymphocyte, **L**; mature thrombocyte, **T**. Wright's stain.